APPLICATION

FOR

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TITLE: IMMUNOGENIC MYCOPLASMA HYOPNEUMONIAE POLYPEPTIDES

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IMMUNOGENIC *MYCOPLASMA HYOPNEUMONIAE* POLYPEPTIDES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. §119(e) of U.S. Application Number 60/392,632, filed June 28, 2002.

BACKGROUND

1. Technical Field

The invention relates to methods and materials involved in protecting an animal against enzootic pneumonia.

2. Background Information

Enzootic pneumonia in swine, also called mycoplasmal pneumonia, is caused by *Mycoplasma hyopneumoniae*. The disease is chronic and non-fatal, affecting pigs of all ages. Although infected pigs show only mild symptoms of coughs and fever, the disease has significant economic impact due to reduced feed efficiency and reduced weight gain. Enzootic pneumonia is transmitted by airborne organisms expelled from the lungs of infected pigs. The primary infection by *M. hyopneumoniae* may be followed by a secondary infection of other *Mycoplasma* species, e.g., *Mycoplasma hyorhinis* and *Mycoplasma flocculare*, as well as other bacterial pathogens.

M. hyopneumoniae infects the respiratory tracts of pigs, colonizing the tracheae, bronchi, and bronchioles. The pathogen produces a ciliostatic factor that causes the cilia lining the respiratory passages to stop beating. Eventually, the cilia degenerate, leaving pigs prone to infection by secondary pathogens. Characteristic lesions of purple to gray areas of consolidation are observed in infected pigs. Surveys of slaughtered pigs revealed lesions in 30 % to 80 %. Results from 37 herds in 13 states indicated that 99 % of the herds had pigs with pneumonia lesions typical of enzootic pneumonia. Therefore, there is a need for effective preventative and treatment measures.

Mycoplasmas vary their surface structure by a complex series of genetic events to present a structural mosaic to the host immune system. Phase switching of surface molecules occurs through a variety of mechanisms such as changes in the number of repetitive units during DNA replication, genomic inversions, transposition events, and/or gene conversion. See, for example, Zhang and Wise, 1997, *Mol. Microbiol.*, 25:859-69; Theiss and Wise, 1997, *J. Bacteriol.*, 179:4013-22; Sachse et al., 2000, *Infect. Immun.*, 68:680-7; Dybvig and Uy, 1994, *Mol. Microbiol.*, 12:547-60; and Lysnyansky et al., 1996, *J. Bacteriol.*, 178:5395-5401. All of the identified phase variable and phase switching genes in mycoplasmas that code for surface proteins are lipoproteins.

SUMMARY

The invention provides materials and methods for protecting an animal from enzootic pneumonia. The invention is based on the discovery of *Mycoplasma hyopneumoniae* nucleic acids that encode cell surface polypeptides that can be used for inducing a protective immune response in an animal susceptible to pneumonia. More specifically, the invention provides purified immunogenic polypeptides of these polypeptides for used to as antigens for illiciting an immune response in an animal, *e.g.* a pig. In addition, the invention also provides isolated nucleic acids encoding these immunogenic polypeptides for use in generating an immune response in an animal. Purified polypeptides and isolated nucleic acids of the invention can be combined with pharmaceutically acceptable carriers for introducing into an animal. The invention also provides materials and methods for determining whether an animal has an antibody reactive to the polypeptides of the invention.

In one aspect, the invention provides a purified immunogenic polypeptide, the amino acid sequence of which comprises at least eight consecutive residues of a sequence selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, and 20. Specifically, the invention provides an immunogenic polypeptide of the invention, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO: 2; an immunogenic polypeptide of the invention, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:4; an immunogenic polypeptide of the invention, the amino acid sequence of which comprises at least eight

consecutive residues of SEQ ID NO:6; an immunogenic polypeptide of the invention, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:8; an immunogenic polypeptide of the invention, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:10; an immunogenic polypeptide of the invention, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:12; an immunogenic polypeptide of the invention, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:14; an immunogenic polypeptide of the invention, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:16; an immunogenic polypeptide of the invention, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:18; an immunogenic polypeptide of the invention, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO: 20.

In another aspect, the invention provides mutants of the above-described immunogenic polypeptides, wherein such mutant polypeptides retain immunogenicity.

Generally, immunogenic polypeptides and immunogenic mutant polypeptides of the invention include at least 8 consecutive residues (e.g., at least 10, 12, 15, 20, or 25) of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, or 20.

In another aspect, the invention provides a composition that includes one or more of the above-described immunogenic polypeptides or immunogenic mutant polypeptides.

In one aspect, the invention provides a method of eliciting an immune response in an animal. Such a method includes introducing a composition comprising the above-described immunogenic polypeptides or immunogenic mutant polypeptides into the animal. Such a composition can be administered orally, intranasally, intraperitoneally, intramuscularly, subcutaneously, or intravenously. A representative animal into which the compositions of the invention can be introduced is a swine.

In another aspect, the invention provides an isolated nucleic acid comprising a nucleotide sequence that encodes an immunogenic polypeptide, the amino acid sequence of which comprises at least eight consecutive residues of a sequence such as SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, or 20. The invention also features mutants of nucleic acids that encode an immunogenic polypeptide. Representative nucleic acids encoding

such immunogenic polypeptides have a nucleotide sequence as shown in SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19, respectively.

Specifically, the invention provides a nucleic acid having a nucleotide sequence encoding an immunogenic polypeptide, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:2. A representative nucleic acid encoding such a polypeptide has the nucleotide sequence of SEQ ID NO:1.

Specifically, the invention provides a nucleic acid having a nucleotide sequence encoding an immunogenic polypeptide, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:4. A representative nucleic acid encoding such a polypeptide has the nucleotide sequence of SEQ ID NO:3.

Specifically, the invention provides a nucleic acid having a nucleotide sequence encoding an immunogenic polypeptide, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:6. A representative nucleic acid encoding such a polypeptide has the nucleotide sequence of SEQ ID NO:5.

Specifically, the invention provides a nucleic acid having a nucleotide sequence encoding an immunogenic polypeptide, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:8. A representative nucleic acid encoding such a polypeptide has the nucleotide sequence of SEQ ID NO:7.

Specifically, the invention provides a nucleic acid having a nucleotide sequence encoding an immunogenic polypeptide, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:10. A representative nucleic acid encoding such a polypeptide has the nucleotide sequence of SEQ ID NO:9.

Specifically, the invention provides a nucleic acid having a nucleotide sequence encoding an immunogenic polypeptide, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:12. A representative nucleic acid encoding such a polypeptide has the nucleotide sequence of SEQ ID NO:11.

Specifically, the invention provides a nucleic acid having a nucleotide sequence encoding an immunogenic polypeptide, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:14. A representative nucleic acid encoding such a polypeptide has the nucleotide sequence of SEQ ID NO:13.

Specifically, the invention provides a nucleic acid having a nucleotide sequence encoding an immunogenic polypeptide, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:16. A representative nucleic acid encoding such a polypeptide has the nucleotide sequence of SEQ ID NO:15.

Specifically, the invention provides a nucleic acid having a nucleotide sequence encoding an immunogenic polypeptide, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:18. A representative nucleic acid encoding such a polypeptide has the nucleotide sequence of SEQ ID NO:17.

Specifically, the invention provides a nucleic acid having a nucleotide sequence encoding an immunogenic polypeptide, the amino acid sequence of which comprises at least eight consecutive residues of SEQ ID NO:20. A representative nucleic acid encoding such a polypeptide has the nucleotide sequence of SEQ ID NO:19.

The invention also provides a vector containing a nucleic acid of the invention. A vector can further include an expression control sequence operably linked to the nucleic acid. The invention additionally provides host cells comprising such vectors. The invention further provides a composition that includes such vectors and a pharmaceutically acceptable carrier.

In yet another aspect, the invention provides a method of eliciting an immune response in an animal. Such a method includes introducing a composition of the invention into the animal. Such compositions can be administered orally, intranasally, intraperitoneally, intramuscularly, subcutaneously, or intravenously. Generally, the animal is a swine.

In still yet another aspect, the invention provides a method of determining whether or not an animal has an antibody reactive to an immunogenic polypeptide of the invention, the method comprising: providing a test sample from the animal; contacting the test sample with the immunogenic polypeptide under conditions permissible for specific binding of the immunogenic polypeptide with the antibody; and detecting the presence or absence of the specific binding. Typically, the presence of specific binding indicates that the animal has the antibody, and the absence of specific binding indicates that the animal does not have the antibody.

Generally, an appropriate test sample is a biological fluid such as blood, nasal fluid, throat fluid, or lung fluid. In some embodiments, the immunogenic polypeptide is attached to a solid support such as a microtiter plate, or polystyrene beads. In some embodiments, the immunogenic polypeptide is labeled. By way of example, the detecting step can be by radioimmunoassay (RIA), enzyme immunoassay (EIA), or enzyme-linked immunosorbent assay (ELISA).

In another aspect, the invention provides a diagnostic kit for detecting the presence of an antibody in a test sample, wherein such an antibody is reactive to an immunogenic polypeptide of the invention. Such a kit can include one or more of the immunogenic polypeptides of the invention.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1 is the nucleic acid sequence encoding C2-mhp210 (SEQ ID NO:1), a P102 paralog from *M. hyopneumoniae* strain 232.

FIG. 2 is the polypeptide sequence of C2-MHP210 (SEQ ID NO:2) from *M. hyopneumoniae* strain 232.

FIG. 3 is the nucleic acid sequence encoding C2-mhp211 (SEQ ID NO:3) from *M. hyopneumoniae* strain 232.

FIG. 4 is the polypeptide sequence of C2-MHP211 (SEQ ID NO:4) from *M. hyopneumoniae* strain 232.

FIG. 5 is the nucleic acid sequence encoding C27-mhp348 (SEQ ID NO:5), a P102 paralog from *M. hyopneumoniae* strain 232.

FIG. 6 is the polypeptide sequence of C27-MHP348 (SEQ ID NO:6) from *M. hyopneumoniae* strain 232.

FIG. 7 is the nucleic acid sequence encoding C28-mhp545 (SEQ ID NO:7) from *M. hyopneumoniae* strain 232.

FIG. 8 is the polypeptide sequence of C28-MHP545 (SEQ ID NO:8) from *M. hyopneumoniae* strain 232.

FIG. 9 is the nucleic acid sequence encoding C28-mhp662 (SEQ ID NO:9) from *M. hyopneumoniae* strain 232.

FIG. 10 is the polypeptide sequence of C28-MHP662 (SEQ ID NO:10) from *M. hyopneumoniae* strain 232.

FIG. 11 is the nucleic acid sequence encoding C28-mhp663 (SEQ ID NO:11), a P102 paralog from *M. hyopneumoniae* strain 232.

FIG. 12 is the polypeptide sequence of C28-MHP663 (SEQ ID NO:12) from *M. hyopneumoniae* strain 232.

FIG. 13 is the nucleic acid sequence encoding C2-mhp036 (SEQ ID NO: 13), a P102 paralog from *M. hyopneumoniae* strain 232.

FIG. 14 is the polypeptide sequence of C2-MPH036 (SEQ ID NO:14) from *M. hyopneumoniae* strain 232.

FIG. 15 is the nucleic acid sequence encoding C2-mhp033 (SEQ ID NO: 15), a partial paralog of P102 from *M. hyopneumoniae* strain 232.

FIG. 16 is the polypeptide sequence of C2-MHP033 (SEQ ID NO:16) from *M. hyopneumoniae* strain 232.

FIG. 17 is the nucleic acid sequence encoding C2-mhp034 (SEQ ID NO: 17), a partial paralog of P102 from *M. hyopneumoniae* strain 232.

FIG. 18 is the polypeptide sequence of C2-MHP034 (SEQ ID NO:18) from *M. hyopneumoniae* strain 232.

FIG. 19 is the nucleic acid sequence encoding C28-mhp545 (SEQ ID NO:19) from *M. hyopneumoniae* strain J.

FIG. 20 is the polypeptide sequence of C28-MHP545 (SEQ ID NO:20) from *M. hyopneumoniae* strain J.

FIG. 21 is the structure of P102 paralogs and their organization in the chromosome.

FIG. 22 shows a map and hydrophilicity plot of P216. The upper panel depicts a schematic diagram of the P216 protein sequence. Asterisks indicate locations of peptides used to clone the gene (left, amino acids 94-105) and used to make antisera specific for P130 (right, amino acids 1654-1668). The arrow indicates the position of the major cleavage event. The gray box indicates the position of the 30-kDa fragment cloned and expressed (amino acids 1043-1226). The inverted filled triangles are locations of tryptophan residues encoded by TGA codons. The hatched boxes are the location of the coiled coil domains. The white box indicates the location of the BNBD (amino acids 1012-1029). The black box represents the transmembrane domain (amino acids 7-30). The lower panel represents the hydrophilicity plot.

DETAILED DESCRIPTION

The following abbreviations are used in this application: aa, amino acid(s); Ab, antibody(ies); bp, base pair(s); CHEF, clamped homogenous electric field; H., *Haemophilus*; kb, kilobase(s) or 1000 bp; Kn, kanamycin; LB, Luria-Bertoni media; M., *Mycoplasma*; mAb, monoclonal Ab; ORF, open reading frame; PCR, polymerase chain reaction; ^{R}, resistant/resistance; Tn, transposon(s); ::, novel junction (fusion or insertion). One letter and three letter code designations for amino acids are given in Table 1.

TABLE 1

Amino Acid Code Designations

Amino Acid	Three letter code	One Letter code	Amino Acid	Three Letter code	One letter code
Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic Acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamic Acid	Glu	E	Serine	Ser	S
Glutamine	Gln	Q	Threonine	Thr	T

Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

M. hyopneumoniae polypeptides and nucleic acids

As used herein, the term "polypeptide" refers to a polymer of three or more amino acids covalently linked by amide bonds. A polypeptide may or may not be post-translationally modified. As used herein, the term "purified polypeptide" refers to a polypeptide preparation that is substantially free of cellular material or other contaminating polypeptides from the cell or tissue source from which the polypeptide is derived, or substantially free of chemical precursors or other chemicals when chemically synthesized. For example, a polypeptide preparation is substantially free of cellular material when the polypeptide is separated from components of the cell from which the polypeptide is obtained or recombinantly produced. Thus, a polypeptide preparation that is substantially free of cellular material includes, for example, a preparation having less than about 30 %, 20 %, 10 %, or 5 % (dry weight) of heterologous polypeptides (also referred to herein as a "contaminating polypeptides"). When a polypeptide is recombinantly produced, the polypeptide is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20 %, 10 %, 5 % of the volume of the polypeptide preparation. When a polypeptide is produced by chemical synthesis, it is preferably substantially free of chemical precursors or other chemicals, i.e., it is separated from chemical precursors or other chemicals that are involved in the synthesis of the polypeptide. Accordingly, such polypeptide preparations have less than about 30 %, 20 %, 10 %, 5 % (by dry weight) of chemical precursors or compounds other than the polypeptide of interest.

As used herein, the term "mutant" refers to a polypeptide, or a nucleic acid encoding a polypeptide, that has one or more conservative amino acid variations or other minor modifications such that (1) the corresponding polypeptide has substantially equivalent function when compared to the wild type polypeptide or (2) an antibody raised against the polypeptide is immunoreactive with the wild-type polypeptide.

The term "conservative variation" denotes the replacement of an amino acid residue by another biologically similar residue, or the replacement of a nucleotide in a nucleic acid sequence such that the encoded amino acid residue does not change or is another biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another hydrophobic residue, or the substitution of one polar residue for another polar residue, such as the substitution of arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

Any *M. hyopneumoniae* strain may be used as a starting material to produce the polypeptides and nucleic acids of the present invention. Suitable strains of *M. hyopneumoniae* may be obtained from a variety of sources, including depositories such as the American Type Culture Collection (ATCC) (Manassas, Va.) and the NRRL Culture Collection (Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill.). *M. hyopneumoniae* strains may also be obtained from lung secretions or tissues from sick animals followed by inoculating suitable culture media.

An immunogenic polypeptide of the present invention can have an amino acid sequence shown in FIG. 2, 4, 6, 8, 10, 12, 14, 16, 18, or 20. Alternatively, an immunogenic polypeptide of the present invention can be a fragment of a polypeptide that has an amino acid sequence shown in FIG. 2, 4, 6, 8, 10, 12, 14, 16, 18, or 20. An immunogenic polypeptide of the invention can be six or more, or preferably eight or more, amino acids in length, but less than the full-length number of amino acids. For example, an immunogenic polypeptide can be 10, 12, 15, 20, 25, 30, or greater than 30 amino acids in length. A polypeptide of the present invention also can be a mutant of a polypeptide having an amino acid sequence shown in FIG. 2, 4, 6, 8, 10, 12, 14, 16, 18, or 20. Mutations at either the amino acid or nucleic acid level may be useful in improving the yield of the polypeptides, their immunogenicity or antigenicity, or their compatibility with various expression systems, adjuvants and modes of administration. Synthetic or recombinant fragments of wild type or mutated polypeptides are

characterized by one or more of the antigenic sites of native *M. hyopneumoniae* polypeptides, the sequences of which are illustrated in FIG. 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20.

The polypeptides of the present invention may be obtained from *M. hyopneumoniae* cells or may be produced in host cells transformed by nucleic acids that encode these polypeptides. Recombinant polypeptides produced from transformed host cells may include residues that are not related to *M. hyopneumoniae.* For example, a recombinant polypeptide may be a fusion polypeptide containing an amino acid portion derived from an expression vector, or other source, in addition to the portion derived from *M. hyopneumoniae.* A recombinant polypeptide may also include a starting methionine. Recombinant polypeptides of the invention display the antigenicity of native *M. hyopneumoniae* polypeptides the sequences of which are illustrated in FIG. 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20.

Nucleic acid sequences encoding full-length polypeptides of the present invention are shown in FIG. 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19. The present invention encompasses nucleic acid sequences, as well as fragments or mutants of these, that encode immunogenic polypeptides, i.e., capable of eliciting antibodies or other immune responses (e.g., T-cell responses of the immune system) that recognize epitopes of the polypeptides having sequences illustrated in FIG. 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20. Hence, nucleic acid sequences of the present invention may encode polypeptides that are full-length polypeptides, polypeptide fragments, and mutant or fusion polypeptides.

The term "nucleic acid" as used herein encompasses RNA and DNA, including cDNA, genomic DNA, and synthetic (e.g., chemically synthesized) DNA. The nucleic acid can be double-stranded or single-stranded. Where single-stranded, the nucleic acid can be the sense strand or the antisense strand. In addition, nucleic acid can be circular or linear.

The term "isolated" as used herein with reference to nucleic acid refers to a naturally-occurring nucleic acid that is not immediately contiguous with both of the sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism from which it is derived. For example, an isolated nucleic acid can be, without limitation, a recombinant DNA

molecule of any length, provided one of the nucleic acid sequences normally found immediately flanking that recombinant DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a recombinant DNA that exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include a recombinant DNA molecule that is part of a hybrid or fusion nucleic acid sequence.

The term "isolated" as used herein with reference to nucleic acid also includes any non-naturally-occurring nucleic acid since non-naturally-occurring nucleic acid sequences are not found in nature and do not have immediately contiguous sequences in a naturally occurring genome. For example, non-naturally-occurring nucleic acid such as an engineered nucleic acid is considered to be isolated nucleic acid. Engineered nucleic acid can be made using common molecular cloning or chemical nucleic acid synthesis techniques. Isolated non-naturally-occurring nucleic acid can be independent of other sequences, or incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or the genomic DNA of a prokaryote or eukaryote. In addition, a non-naturally-occurring nucleic acid can include a nucleic acid molecule that is part of a hybrid or fusion nucleic acid sequence.

It will be apparent to those of skill in the art that a nucleic acid existing among hundreds to millions of other nucleic acid molecules within, for example, cDNA or genomic libraries, or gel slices containing a genomic DNA restriction digest is not to be considered an isolated nucleic acid.

The term "exogenous" as used herein with reference to nucleic acid and a particular cell refers to any nucleic acid that does not originate from that particular cell as found in nature. Thus, non-naturally-occurring nucleic acid is considered to be exogenous to a cell once introduced into the cell. It is important to note that non-naturally-occurring nucleic acid can contain nucleic acid sequences or fragments of nucleic acid sequences that are found in nature provided the nucleic acid as a whole does

not exist in nature. For example, a nucleic acid molecule containing a genomic DNA sequence within an expression vector is non-naturally-occurring nucleic acid, and thus is exogenous to a cell once introduced into the cell, since that nucleic acid molecule as a whole (genomic DNA plus vector DNA) does not exist in nature. Thus, any vector, autonomously replicating plasmid, or virus (e.g., retrovirus, adenovirus, or herpes virus) that as a whole does not exist in nature is considered to be non-naturally-occurring nucleic acid. It follows that genomic DNA fragments produced by PCR or restriction endonuclease treatment as well as cDNAs are considered to be non-naturally-occurring nucleic acid since they exist as separate molecules not found in nature. It also follows that any nucleic acid containing a promoter sequence and polypeptide-encoding sequence (e.g., cDNA or genomic DNA) in an arrangement not found in nature is non-naturally-occurring nucleic acid.

Nucleic acid that is naturally occurring can be exogenous to a particular cell. For example, an entire chromosome isolated from a cell of person X is an exogenous nucleic acid with respect to a cell of person Y once that chromosome is introduced into Y's cell.

Recombinant nucleic acid molecules that are useful in preparing the aforementioned polypeptides are also provided. Preferred recombinant nucleic acid molecules include, without limitation, (1) those having nucleic acid sequences illustrated in FIG. 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19; (2) cloning or expression vectors containing sequences encoding recombinant polypeptides of the present invention; (3) nucleic acid sequences that hybridize to those sequences that encode *M. hyopneumoniae* polypeptides of the invention; (4) degenerate nucleic acid sequences that encode polypeptides of the invention.

Nucleic acids of the invention may be inserted into any of a wide variety of expression vectors by a variety of procedures, generally through use of an appropriate restriction endonuclease site. Suitable vectors include, for example, vectors consisting of segments of chromosomal, non-chromosomal and synthetic nucleic acid sequences, such as various known derivatives of SV40; known bacterial plasmids, e.g., plasmids from *E. coli* including col E1, pCR1, pBR322, pMB9 and their derivatives; wider host range plasmids, e.g., RP4; phage DNAs, e.g., the numerous derivatives of phage λ, e.g., NM 989, and other DNA phages such as M13 or filamentous single stranded DNA phages;

yeast plasmids such as the 2μ plasmid or derivatives thereof; viral DNA such as baculovirus, vaccinia, adenovirus, fowl pox virus, or pseudorabies; and vectors derived from combinations of plasmids and phage DNAs, such as plasmids which have been modified to employ phage DNA or other expression control sequences.

Within each specific cloning or expression vector, various sites may be selected for insertion of the nucleic acids of this invention. These sites are usually designated by the restriction endonuclease that cuts them, and there are various known methods for inserting nucleic acids into these sites to form recombinant molecules. These methods include, for example, dG-dC or dA-dT tailing, direct ligation, synthetic linkers, exonuclease and polymerase-linked repair reactions followed by ligation, or extension of the nucleic acid strand with DNA polymerase and an appropriate single-stranded template followed by ligation. It is to be understood that a cloning or expression vector useful in this invention need not have a restriction endonuclease site for insertion of the chosen nucleic acid fragment, and that insertion may occur by alternative means.

For expression of the nucleic acids of this invention, these nucleic acid sequences are operatively linked to one or more expression control sequences in the expression vector. Such operative linking, which may be effected before or after the chosen nucleic acid is inserted into a cloning vehicle, enables the expression control sequences to control and promote the expression of the inserted nucleic acid.

Any of a wide variety of expression control sequences--sequences that control the expression of a nucleic acid when operatively linked to it--may be used in these vectors to express the nucleic acid sequences of this invention. Such useful expression control sequences include, for example, the early and late promoters of SV40, the lac or trp systems, the TAC or TRC system, the major operator and promoter regions of λ, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, and other sequences known to control the expression of genes in prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. The expression vector also includes a non-coding sequence for a ribosome-binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. In mammalian cells, it is additionally

possible to amplify the expression units by linking the gene to that coding for dehydrofolate reductase and applying a selection to host Chinese hamster ovary cells.

The vector or expression vehicle, and in particular, the sites chosen therein for insertion of the selected nucleic acid fragment, and the expression control sequence employed in this invention are determined by a variety of factors, e.g., number of sites susceptible to a particular restriction enzyme, size of the polypeptide to be expressed, expression characteristics such as the location of start and stop codons relative to the vector sequences, and other factors recognized by those of skill in the art. The choice of a vector, expression control sequence, and/or insertion site are determined by a balance of these factors, as not all selections are equally effective for a given case.

The recombinant nucleic acid molecule containing the desired coding sequence operatively linked to an expression control sequence may then be employed to transform a wide variety of appropriate hosts so as to permit such hosts (transformants) to express the coding sequence, or fragment thereof, and to produce the polypeptide, or portion thereof, for which the hybrid nucleic acid encodes. The recombinant nucleic acid molecule may also be employed to transform a host so as to permit that host on replication to produced additional recombinant nucleic acid molecules as a source of *M. hyopneumoniae* coding sequences and fragments thereof.

A wide variety of hosts are also useful in producing polypeptides and nucleic acids of this invention. These hosts include, for example, bacteria such as *E. coli*, *Bacillus* and *Streptomyces*, fungi such as yeasts, and animal or plant cells in tissue culture. The selection of an appropriate host for these uses is controlled by a number of factors. These include, for example, compatibility with the chosen vector, toxicity of the co-products, ease of recovery of the desired polypeptide, expression characteristics, biosafety and costs. No absolute choice of host may be made for a particular recombinant nucleic acid molecule or polypeptide from any of these factors alone. Instead, a balance of these factors is applied with the realization that not all hosts may be equally effective for expression of a particular recombinant nucleic acid molecule.

It is also understood that the nucleic acid sequences that are inserted at the selected site of a cloning or expression vector may include nucleotides that are not part of the actual coding sequence for the desired polypeptide or may include only a fragment of

the entire coding sequence for that polypeptide. It is only required that whatever DNA sequence is employed, the transformed host produces a polypeptide having the antigenicity of native *M. hyopneumoniae* polypeptides.

For example, in an expression vector of this invention, a nucleic acid of this invention may be fused in the same reading frame to a portion of a nucleic acid sequence coding for at least one eukaryotic or prokaryotic carrier polypeptide or a nucleic acid sequence coding for at least one eukaryotic or prokaryotic signal sequence, or combinations thereof. Such constructions may aid in expression of the desired nucleic acid sequence or improve purification, permit secretion, and preferably maturation of the desired polypeptide from the host cell. The nucleic acid sequence may alternatively include an ATG start codon, alone, or together with other codons, fused directly to the sequence encoding the first amino acid of a desired polypeptide. Such constructions enable the production of, for example, a methionyl or other peptidyl polypeptide that is part of this invention. This N-terminal methionine or peptide may then be cleaved intracellularly or extracellularly by a variety of known processes or the polypeptide used together with the methionine or other fusion attached to it in the compositions and methods of this invention.

The appropriate nucleic acid sequence present in the vector when introduced into a host may express part or only a portion of the polypeptide that is encoded, it being sufficient that the expressed polypeptide be capable of eliciting an antibody or other immune response that recognizes an epitope of the amino acid sequence depicted in FIG. 2, 4, 6, 8, 10, 12, 14, 16, 18, or 20. For example, in employing *E. coli* as a host organism, the UGA codon is a stop codon so that the expressed polypeptide may only be a fragment of the polypeptide encoded by the vector, and therefore, it is generally preferred that all of the UGA codons in the appropriate nucleic acid sequence be converted into non-stop codons. Alternatively, an additional nucleic acid sequence that encodes a t-RNA that translates the UGA codon into a tryptophan residue can be introduced into the host.

The polypeptide expressed by the host transformed by the vector may be harvested by methods known to those skilled in the art, and used for protection of a non-human animal such as swine, cattle, etc. against enzootic pneumonia caused by *M. hyopneumoniae*. The polypeptide is used in an amount effective to provide protection

against enzootic pneumonia caused by *M. hyopneumoniae* and may be used in combination with a suitable physiologically acceptable carrier as described below.

Detecting M. hyopneumoniae

The polypeptides of the present invention may also be used as antigens for diagnostic purposes to determine whether a biological test sample contains *M. hyopneumoniae* antigens or antibodies to these antigens. Such assays for *M. hyopneumoniae* infection in an animal typically involve incubating an antibody-containing biological sample from an animal suspected of having such a condition in the presence of a detectably labeled polypeptide of the present invention, and detecting binding. The immunogenic polypeptide is generally present in an amount that is sufficient to produce a detectable level of binding with antibody present in the antibody-containing sample.

Thus, in this aspect of the invention, the polypeptide may be attached to a solid phase support, e.g., a microtiter plate, which is capable of immobilizing cells, cell particles or soluble polypeptides. The support may then be washed with suitable buffers followed by treatment with the sample from the animal. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. Labeled polypeptide is added and the support is washed a third time to remove unbound labeled polypeptide. The amount of bound label on said solid support may then be detected by conventional means.

By "solid phase support" is intended any support capable of binding antigen or antibodies. Well-known supports, or carriers, include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses (especially nitrocellulose), polyacrylamides, agarose, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as for example, a sheet or test strip. Preferred supports include polystyrene beads.

M. hyopneumoniae specific antibody can be detectably labeled by linking the same to an enzyme and using it in an enzyme immunoassay (EIA), or enzyme-linked immunosorbent assay (ELISA). This enzyme, in turn, when later exposed to its substrate, will react with the substrate in such a manner as to produce a chemical moiety that can be detected, for example, by spectrophotometric, fluorometric or by visual means. Enzymes that can be used to detectably label the *M. hyopneumoniae* specific antibody include, but are not limited to, horseradish peroxidase, malate dehydrogenase, staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoa/nylase and acetylcholinesterase.

Detection may be accomplished using any of a variety of immunoassays. For example, by radioactively labeling the recombinant protein, it is possible to detect antibody binding through a radioimmunoassay (RIA). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for the purpose of the present invention include ^{3}H, ^{125}I, ^{131}I, ^{35}S, and ^{14}C, preferably ^{125}I.

It is also possible to label the recombinant polypeptide with a fluorescent compound. When the fluorescently labeled polypeptide is exposed to light of the proper wavelength, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine. The polypeptide can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the protein using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediamine-tetraacetic acid (EDTA).

The polypeptide also can be detectably labeled by coupling it to a chemiluminescent or bioluminescent compound. The presence of the chemiluminescent-tagged polypeptide is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the

efficiency of the chemiluminescent reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Detection of the label may be accomplished by a scintillation counter, for example, if the detectable label is a radioactive gamma emitter, or by a fluorometer, for example, if the label is a fluorescent material. In the case of an enzyme label, the detection can be accomplished by colorimetric methods that employ a substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

The detection of foci of detectably labeled antibodies is indicative of a disease or dysfunctional state and may be used to measure *M. hyopneumoniae* in a sample. The absence of such antibodies or other immune response indicates that the animal has been neither vaccinated nor infected. For the purposes of the present invention, the bacterium that is detected by this assay may be present in a biological sample. Any sample containing it can be used, however, one of the benefits of the present diagnostic invention is that invasive tissue removal may be avoided. Therefore, preferably, the sample is a biological fluid such as, for example, blood, or nasal, throat or lung fluid, but the invention is not limited to assays using these samples.

In situ detection may be accomplished by removing a histological specimen from an animal, and providing the combination of labeled antibodies of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of *M. hyopneumoniae* but also the distribution of it in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Alternatively, a sample (e.g., a fluid or tissue sample) may be tested for the presence of a coding sequence for a *M. hyopneumoniae* polypeptide of the invention by reaction with a recombinant or synthetic nucleic acid sequence contained within the sequence shown in FIG. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, or any RNA sequence equivalent

to this nucleic acid sequence. The absence of the coding sequence indicates that the animal has been neither vaccinated nor infected. This test involves methods of synthesis, amplification, or hybridization of nucleic acid sequences that are known to those skilled in the art. See, for example, Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; PCR, A Practical Approach, Vols 1 & 2, McPherson et al. (eds.), Oxford University Press, 1992 and 1995; and PCR Strategies, Innis (ed.), Academic Press, 1995, herein incorporated by reference.

Compositions

The present invention also contemplates a composition (e.g., a vaccine) comprising the recombinant polypeptides of the present invention, or nucleic acid sequences encoding these polypeptides, for immunizing or protecting non-human animals, preferably swine, against *M. hyopneumoniae* infections, particularly enzootic pneumonia. The terms "protecting" or "protection" when used with respect to the composition for enzootic pneumonia described herein means that the composition prevents enzootic pneumonia caused by *M. hyopneumoniae* and/or reduces the severity of the disease. When a composition elicits an immunological response in an animal, the animal is considered seropositive, i.e., the animal produces a detectable amount of antibodies against a polypeptide of the invention. Methods for detecting an immunological response in an animal are well known.

Compositions generally include an immunologically effective dosage of a polypeptide of the invention. An "immunologically effective" dosage is an amount that, when administered to an animal, elicits an immunological response in the animal but does not cause the animal to develop severe clinical signs of an infection. An animal that has received an immunologically effective dosage is an inoculated animal or an animal containing an inoculant of an immunologically effective amount of a polypeptide of the invention. Immunologically effective dosages can be determined experimentally and may vary according to the type, size, age, and health of the animal vaccinated. The vaccination may include a single inoculation or multiple inoculations. Other dosage schedules and amounts, including vaccine booster dosages, may be useful.

The composition can be employed in conjunction with a carrier, which may be any of a wide variety of carriers. Representative carriers include sterile water, saline, buffered solutions, mineral oil, alum, and synthetic polymers. Additional agents to improve suspendability and dispersion in solution may also be used. The selection of a suitable carrier is dependent upon the manner in which the composition is to be administered. The composition is generally employed in non-human animals that are susceptible to enzootic pneumonia, in particular, swine.

The composition may be administered by any suitable method, such as intramuscular, subcutaneous, intraperitoneal or intravenous injection. Alternatively, the composition may be administered intranasally or orally, such as by mixing the active components with feed or water, or providing a tablet form. Methods such as particle bombardment, microinjection, electroporation, calcium phosphate transfection, liposomal transfection, and viral transfection are particularly suitable for administering a nucleic acid. Nucleic acid compositions and methods of their administration are known in the art, and are described in U.S. Patent Nos. 5,836,905; 5,703,055; 5,589,466; and 5,580,859, which are herein incorporated by reference. Other means for administering the composition will be apparent to those skilled in the art from the teachings herein; accordingly, the scope of the invention is not limited to a particular delivery form.

The composition may also include active components or adjuvants (e.g., Freund's incomplete adjuvant) in addition to the antigen(s) or fragments hereinabove described. Adjuvants may be used to enhance the immunogenicity of an antigen. Among the adjuvants that may be used are oil and water emulsions, complete Freund's adjuvant, incomplete Freund's adjuvant, *Corynebacterium parvum, Hemophilus*, *Mycobacterium butyricum*, aluminum hydroxide, dextran sulfate, iron oxide, sodium alginate, Bacto-Adjuvant, certain synthetic polymers such as poly amino acids and co-polymers of amino acids, saponin, iota carrageenan, Regressin™, Avridine™, *Mannite monooleate*, paraffin oil, and muramyl dipeptide.

Nucleic acid or polypeptide compositions or vaccines as described herein can be combined with packaging materials including instructions for their use to be sold as articles of manufacture or kits. Components and methods for producing articles of manufactures are well known. The articles of manufacture may combine one or more

vaccines (e.g., nucleic acid or polypeptide) as described herein. Instructions describing how a vaccine is effective for preventing the incidence of a *M. hyopneumoniae* infection, preventing the occurrence of the clinical signs of a *M. hyopneumoniae* infection, ameliorating the clinical signs of a *M. hyopneumoniae* infection, lowering the risk of the clinical signs of a *M. hyopneumoniae* infection, lowering the occurrence of the clinical signs of a *M. hyopneumoniae* infection and/or spread of *M. hyopneumoniae* infections in animals may be included in such kits.

Conveniently, vaccines of the invention may be provided in a pre-packaged form in quantities sufficient for a protective dose for a single animal or for a pre-specified number of animals in, for example, sealed ampoules, capsules or cartridges.

Application of the teachings of the present invention to a specific problem or environment is within the capabilities of one having ordinary skill in the art. Examples of the products and processes of the present invention appear in the following examples.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

A. P102 AND PARALOGS THEREOF

Example A.1 – *Mycoplasma* strains

Mycoplasmas hyopneumoniae strains used included the 232, J, and Beaufort. The source and culture conditions used to grow *M. hyopneumoniae* are as described in Scarman et al. (1997) *Microbiology* 143:663-673.

Example A.2 – Cloning of the gene encoding P102

The gene encoding P102 was obtained by polymerase chain reaction (PCR) and cloned into pTrcHis (Invitrogen). The oligonucleotides TH130 and TH131 were used to amplify the region encoding amino acids 33 to 887 of P102 from pISM1217 as described in Hsu and Minion ((1998) *Infect. Immun.* 66:4762-4766). The PCR product having 5' *Bam*HI and 3' *Pst*I restriction enzyme sites was digested sequentially with *Bam*HI and *Pst*I, gel purified, and ligated into *Bam*HI/*Pst*I-digested pTrcHis plasmid DNA. The ligation mixture was transformed into CSH50 *Escherichia coli*, and transformants were

selected for ampicillin resistance (100 μg per mL). The resulting plasmid was sequenced with primer SA1528 to confirm the insertion and orientation of the insert.

Site directed mutagenesis was performed on the insert sequence to remove TGA codons, which code for tryptophan in *Mycoplasmas*. Directed mutagenesis was performed using the Stratagene QuikChange Site-Directed Mutagenesis Kit (Stratagene, CA) according to the manufacturer's instructions. Five TGA codons in the cloned sequence were changed to TGG using the following primer pairs:

P102.2f: 5'-GAT AAT TTT AAA AAA TGG TCG GCA AAA ACA GTT TTA ACT GCT GCC-3' (SEQ ID NO:21);

P102.2r: 5'-GGC AGC AGT TAA AAC TGT TTT TGC CGA CCA TTT TTT AAA ATT ATC-3' (SEQ ID NO:22);

P102.3f: 5'-GAA AGA GGA AGT AAT TGG TTT TCA CGA CTT GAA AGA GC-3' (SEQ ID NO:23);

P102.3r: 5'-GCT CTT TCA AGT CGT GAA AAC CAA TTA CTT CCT CTT TC-3' (SEQ ID NO:24);

P102.4f: 5'-CTA AAA TTC TAA AAT CCT GGC TTG AAA CAA ATC TTC AAG GC-3' (SEQ ID NO:25);

P102.4r: 5'-GCC TTG AAG ATT TGT TTC AAG CCA GGA TTT TAG AAT TTT AG-3' (SEQ ID NO:26);

P102.5f: 5'-GCC TCT CTG ATT ATT GGT ATG GAT CTC CGA ATT C-3' (SEQ ID NO:27);

P102.5r: 5'-GAA TTC GGA GAT CCA TAC CAA TAA TCA GAG AGG C-3' (SEQ ID NO:28);

P102.6f: 5'-GGG ACA AGC ATT TGG ACA GCT TTT AAT TTC G-3' (SEQ ID NO:29);

P102.6r: 5'-CGA AAT TAA AAG CTG TCC AAA TGC TTG TCC C-3' (SEQ ID NO:30).

E. coli XL1-Blue MRF' was the recipient for each mutagenesis step. To confirm the sequence and the single-base changes, and to determine whether errors were introduced during the cloning and mutagenesis steps, the final product was sequenced using the primers:

P102.2-SEQ: 5'-TCC GAC GAT GAC GAT AAG-3' (SEQ ID NO:31);

P102.5-SEQ: 5'-TGG AAA ATT AGT TCT TGG-3' (SEQ ID NO:32);

P102.6-SEQ: 5'-AGT TTC CAC TTC ATC GCC-3' (SEQ ID NO:33).

The final construct was designated pISM1316.6.

Example A.3 – Expression and purification of P102

Plasmid pISM1316.6 was transformed into *E. coli* ER1458 (*F- Δ(lac)U169 lon-100 hsdR araD139 rpsL(StrR) supF mcrA trp+ zjj202::Tn10(TetR) hsdR2(rk-mk+) mcrB1*), a Lon protease mutant, in preparation for protein expression. An overnight culture was diluted 1:10 into fresh superbroth medium (per liter; 32 g Bacto tryptone, 20 g yeast extract, 5 g sodium chloride, pH 7.3) containing 1 mM isopropyl thiogalactopyranoside (IPTG) and protease inhibitor cocktail (Sigma P8848) at a 1:200 dilution. The culture was incubated for 5 hours at 30°C with shaking. The cells were collected by centrifugation and resuspended in TS buffer (10 mM Tris, 100 mM sodium chloride, pH 7.4) plus 8 M urea and 2 mg/mL of lysozyme. After incubating for 30 minutes on ice, the suspension was frozen in a dry ice ethanol bath and passed sequentially through three freeze-thaw cycles. The chromosomal DNA was sheared by passing the suspension through an 18-gauge needle, and insoluble cellular debris was removed by centrifugation. The final solution was passed through a Talon Metal Affinity Resin (Clontech Laboratories, Inc., CA) column. The column was washed with 10 column volumes of TS buffer containing 10 mM imidazole. The bound protein was eluted with TS buffer containing 500 mM imidazole, and the column eluent was dialyzed overnight against phosphate buffered saline (10 mM Na_2HPO_4, 100 mM NaCl, pH 7.4). Purity of the protein preparations was assessed by sodium dodecyl sulfate gel electrophoresis and by Western blotting using 6xHis monoclonal antibody (Clontech).

Example A.4 – Generation of P102 antisera

Mice were immunized with 10 μg of purified P102 mixed with 200 μL of Freund's incomplete adjuvant, and on day 21, second dosages were given. Ascites were developed by the introduction of Sp2 myeloma cells using the method of Luo and Lin ((1997) *BioTechniques* 23:630-632), and ascites fluid was aliquoted and stored at –70°C. Antibody specificity was tested by immunoblot analysis using purified P102 protein and *M. hyopneumoniae* whole antigen.

Example A.5 – Immunoelectron microscopic analysis of immunogold-labeled cell sections

To determine if P102 is surface exposed or associated with the P97 cilium adhesin, monospecific polyclonal anti-P102 antiserum was used in the following immunoelectron microscopic studies to determine the location of P102 in the *Mycoplasma* cell.

M. hyopneumoniae strains 90-1 and 60-3 were grown in modified Friis media (Friis (1971) *Acta Vet. Scand.* 12:69-79) until mid log phase as described (Hsu *et al.* (1997) *J. Bacteriol.* 179:1317-1323). The cells were pelleted by centrifugation and washed once with phosphate buffered saline (PBS) by centrifugation. Cells were resuspended in PBS and then reacted with either anti-P102 ascite fluid diluted 1:50, or F1B6 cell culture supernatant (Zhang et al. (1995) *Infect. Immun.* 63:1013-1019) diluted 1:10, overnight at 4°C. The next day, cells were washed five times with PBS and then reacted for 30 minutes at room temperature with goat anti-mouse IgG + IgM labeled with 10 nm gold particles (EY Laboratories, Inc., San Mateo, Calif.) diluted 1:25. The cells were then washed five times with PBS and pelleted by centrifugation.

The final cell pellets were fixed with 3 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C overnight. The pellets were washed three times, 15 minutes each time, with 0.1 M sodium cacodylate buffer and post fixed with 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 hours at room temperature. The pellets were then washed with distilled water, passed through an acetone series and embedded in Embed 812 and Araldite (Electron Microscopy Sciences, Fort Washington, PA).

For tracheal sections, *Mycoplasma*-free pigs were inoculated intratracheally with *M. hyopneumoniae* strain 232 as described in Thacker et al. ((1997) Potentiation of PRRSV pneumonia by dual infection with *Mycoplasma hyopneumoniae*. In *Conference of Research Workers in Animal Diseases*. Ellis, R.P. (ed.) Chicago, IL: Iowa State University Press, pp. 190). At 10 and 21 days, pigs were sacrificed, and tracheas were removed. One cm blocks of tissue were fixed with 1% glutaraldehyde overnight, dehydrated in an acetone series and embedded as above. Thick (1-2 μm) sections were stained with methylene blue polychrome and examined by microscopy for regions containing ciliated epithelium. Thin sections (80-90 nm) were then prepared for labeling. For some studies, cells grown *in vitro* were embedded and sectioned prior to staining. The sections were pretreated with ammonium chloride (1%) for 1 hour, 0.05 M glycine in PBS for 15 minutes, and blocked for 30 minutes in 2% fish gelatin + 2% bovine serum albumin in TS buffer (10 mM Tris, 100 mM NaCl, pH 7.5). Primary antibodies were diluted (1:50) in TS buffer and reacted with sections for 30 minutes at room temperature. The sections were washed six times with TS buffer, and then incubated with goat anti-mouse IgG + IgM labeled with 10 nm gold particles (diluted 1:2) for 15 minutes at room temperature. Both primary antibodies and the conjugate were diluted and centrifuged briefly (12,000 xg for 5 minutes) to remove gold aggregates prior to use. The sections were then washed six times with TS buffer, dried, contrasted with osmium vapors for 2 minutes, and stained with uranyl acetate-lead citrate. The sections were examined on a Hitachi 500 electron microscope at 75 kV.

In *in vitro* grown cells, gold particles were found external to the cells and were primarily associated with the extracellular matrix. Similar results were observed for cells that were stained before or after fixation and sectioning. Occasionally, particles were seen associated with the cell surface, and in rare cases, particles were seen intracellularly. In cells associated with swine cilia, however, gold particles were seen at high concentration intracellularly. P102 was also found in association with swine cilia, often in aggregates or at high concentrations. The extracellular matrix that was so prominent in broth grown cells was not evident in sections of infected swine epithelia.

Example A.6 – Two-dimensional electrophoresis

Two-dimensional gel electrophoresis (2-DGE) was carried out essentially as described by Guerreiro et al. ((1997) *Mol. Plant Microbe Interact.*, 10:506-16). First dimension immobilized pH gradient (IPG) strips (180 mm, linear and non-linear pH 3-10 and linear pH 4-7 and 6-11; Amersham Pharmacia Biotech, Uppsala, Sweden) were prepared for focusing by submersion in hydration buffer (8 M urea, 0.5% wt/vol CHAPS, 0.2% wt/vol DTT, 0.52% wt/vol Bio-Lyte and a trace of bromophenol blue) overnight. *M. hyopneumoniae* whole cell protein (100 μg for analytical gels, 0.5-1.0 mg for preparative gels and immunoblots) was diluted with sample buffer (8 M urea, 4% w/v CHAPS, 1% w/v DTT, 0.8% w/v Bio-Lyte 3-10, 35 mM Tris, and 0.02% w/v bromophenol blue) to a volume of 50 to 100 μL for application to the anodic end of each IPG strip. Isoelectric focusing was performed with a Multiphor II electrophoresis unit (Pharmacia) for 200 kVh at 20°C except for pH 6-11 strips, which were electrophoresed for 85kVh. IEF strips were reduced and alkylated in Tris-HCl (0.5 M, pH 6.8) containing 6 M urea, 30% w/v glycerol, 2% w/v sodium dodecyl sulfate (SDS), 2% w/v DTT and 0.02% bromophenol blue. Equilibrated strips were placed onto Pharmacia ExcelGels (T = 12 to 14% acrylamide) for SDS-PAGE using the Multiphor II. Electrophoretic conditions consisted of 200 Volts for 1.5 hours followed by 4 hours at 600 Volts at 5 °C. Gels were stained in Coomassie Blue R-250 (Bio-Rad, Hercules, CA), and proteins were transferred to polyvinylidene difluoride (PVDF) membranes using a Hoefer TE70 Series SemiPhor Semi-Dry Transfer Unit (Amersham Pharmacia Biotech, Uppsala, Sweden). The transfer was carried out for 1.5 hours at maximum voltage and a current measured by multiplying the area of the gel (cm^2) by 0.8 mA.

Example A.7 – Post-separation analyses

Protein spots were excised from gels using a sterile scalpel and placed in a 96 well tray. Gel pieces were washed with 50 mM ammonium bicarbonate/100% acetonitrile (60:40 v/v) and then dried in a Speed Vac (Savant Instruments, Holbrook, NY) for 25 minutes. Gel pieces were then hydrated in 12 μL of 12 ng μL^{-1} sequencing grade modified trypsin (Promega, Madison, WI) for 1 hour at 4°C. Excess trypsin solution was removed and the gel pieces immersed in 50 mM ammonium bicarbonate and

incubated overnight at 37°C. Eluted peptides were concentrated and desalted using C_{18} Zip-Tips™ (Millipore Corp., Bedford, MA.). The peptides were washed on column with 10 µL of 5% formic acid. The bound peptides were eluted from the Zip-Tip™ in matrix solution (10 mg mL^{-1} α-cyano-4-hydroxycinnamic acid [Sigma] in 70% acetonitrile) directly onto the target plate. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) mass spectra were acquired using either a PerSeptive Biosytems Voyager DE-STR (Framingham, MA.) or a Micromass TofSpec2E (Micromass, Manchester UK). Both instruments were equipped with 337 nm nitrogen lasers. All spectra were obtained in reflectron/delayed extraction mode, averaging 256 laser shots per sample. Two-point internal calibration of spectra was performed based upon internal porcine trypsin autolysis peptides (842.5 and 2211.10 $[M+H]^+$ ions). A list of monoisotopic peaks corresponding to the mass of generated tryptic peptides was used to search a modified translated version of the *M. hyopneumoniae* genome. Successful identifications were based on the number of matching peptide masses and the percentage sequence coverage afforded by those matches. N-terminal Edman sequencing was performed as previously described (Nouwens et al., 2000).

Example A.8 – P102 is surface expressed

To generate a P102 specific antibody, recombinant P102 protein was expressed in *E. coli* and then purified as follows. The coding sequence for P102 was obtained from plasmid pISM1217, which contained the entire sequence of P102 (Hsu and Minion (1998) *Infect. Immun.* 66:4762-4766). The region of the coding sequence encoding amino acids 33-887 was amplified by PCR using primers having *Bam*HI and *Pst*I restriction sites at the 5' termini to enable cloning into pTrcHis. The resulting construct was designated pISM1249. To allow for expression of the coding sequence in *E. coli*, the TGA codons in the pISM1249 sequence were altered by site-directed mutagenesis to TGG codons. The final construct pISM1316.6 was sequenced to confirm these changes and to check for errors introduced by PCR during the mutagenesis step.

Expression of the cloned sequence in pISM1316.6 resulted in a poly-histidine-tagged protein of about 100 kDa. Expression levels of P102 were low in *E. coli* despite the removal of the opal (TGA) stop codons. A Talon Metal Affinity Resin column was

used to remove contaminating *E. coli* proteins during purification. Mouse hyperimmune antiserum raised against this recombinant protein was used in immunoblot analysis of *M. hyopneumoniae* whole cells. The anti-P102 antiserum showed three bands indicating either the presence of cross-reactive proteins or that P102 was being proteolytically processed. Trypsin treatment of whole cells followed by immunoblot and development with the anti-P102 antiserum showed that P102 was located on the membrane surface; all immunoreactive bands were sensitive to trypsin.

Example A.9 – P102 paralogs are found throughout the *M. hyopneumoniae* genome

Hybridization studies indicated that P102 or P102-related sequences may exist in multiple copies in the genome of *M. hyopneumoniae* (Hsu et al. (1997) *J. Bacteriol.* 179:1317-1323). Genome sequencing studies have identified four distinct paralogs of P102 (C2-mhp210, C27-mhp348, C28-mhp663, and C2-mhp036) and two partial paralogs (C2-mhp033 and C2-mhp034) scattered throughout the chromosome (Fig. 21). Further analysis of the genome sequence of *M. hyopneumoniae* revealed additional open reading frames with varying homologies to P102. Each of these appeared to be a fusion with a second gene, while the original P102 sequence had undergone significant evolution. Also, each paralog was part of a two-gene genetic structure, possibly organized into operons. In every case, the P102 paralog was the second or downstream gene. DNA sequence analysis of each of the P102 paralogs showed that homology to P102 was low, but amino acid homology was much higher. The amino acid sequences of the P102 paralogs are shown in Figures 2, 6, 12, 14, 16,18, and 20.

Example A.10 – Biotin labeling of surface accessible proteins identified molecules belonging to a multi-gene family

Studies were undertaken to identify all of the surface accessible proteins in *M. hyopneumoniae* recognized by convalescent and hyperimmune swine sera. By combining surface biotinylation, two-dimensional immunoblotting, genomic and proteomic analysis, a subset of these surface molecules was mapped to the genome sequence of *M. hyopneumoniae.*

Initially, two-dimensional gel electrophoresis of biotinylated proteins identified groups of proteins that were surface exposed, highly expressed, and appeared to resolve along the pI gradient as a series of spots. The molecular masses of many of these proteins ranged from 40 to 130 kDa. Many of these proteins were recognized by convalescent and hyperimmune swine sera. This suggests that these proteins were expressed during *M. hyopneumoniae* infection and evoked an accompanying immune response.

Tryptic fragments of individual protein spots were analyzed by peptide mass fingerprinting, and the spectra matched to theoretical trypsin cleavage products generated from the *M. hyopneumoniae* genome database. Some of the spots of different molecular masses mapped to the same single copy gene.

Example A.11 – Peptide mass fingerprinting and biotinylation studies show that P102 paralogs are expressed

Many of the proteins identified by biotinylation and peptide mass fingerprinting were related to products from the cilium adhesion operon (Hsu and Minion (1998) *Infect. Immun.* 66:4762-4766). In addition to the cilium adhesin P97, gene products representing P102 and related proteins were identified.

A.12 – Results

Results indicated that there were a surprising number of P102 paralogs that were all expressed and located on the surface of the organism. Some of the P102 paralogs had a greater degree of sequence identity with P97, while other P102 paralogs did not. None of the sequences surrounding the P102 paralogs were similar, which suggests that the P102 genes duplicated and moved independently of surrounding sequences. Differential staining of *in vitro*-grown and *in vivo*-grown organisms was observed, further suggesting that P102 might be involved in the hyperimmune-like responses seen during infection.

B. P216 STUDIES

Example B.1 – *Mycoplasma* strains and culture

The source and culture conditions used to grow *M. hyopneumoniae* strains J, Beaufort and 232 are as described in Scarman et al. ((1997) *Microbiology* 143:663-673). *Mycoplasmas* were harvested by centrifugation at 10,000 x*g*, washed three times with TS buffer (10 mM Tris, 150 mM NaCl, pH 7.5), and the final cell pellets were frozen at -20°C until use.

Example B.2 – Preparative electrophoresis

Preliminary vaccine trials in swine immunised with size-fractionated antigens of *M. hyopneumoniae* indicated that antigen pools residing in two fractions, fractions 2 (85-150 kDa) and 3 (70-85 kDa), provided limited protection against a virulent challenge (Djordjevic et. al (1997) *Aust Vet J* 75:504-511). To determine the amino acid sequences of proteins residing in these molecular mass fractions, whole cell lysates of *M. hyopneumoniae* J strain were separated using 5-7% polyacrylamide resolving columns each with a 4% stacking gel using a BioRad 491 Prep Cell as described in Scarman et al. ((1997) *Microbiology* 143:663-673). Proteins corresponding to those defined for fractions 2 and 3 were pooled, concentrated by filtration, and resuspended in PBS. Protein fractions were digested with trypsin, separated using electrophoresis on precast 8-15% gradient Tricine gels (Novex), and then blotted onto PVDF membrane (BioRad, California, USA) (Towbin et al. (1979) *Proc. Natl. Acad. Sci. USA.* 76:4350-4354). Protein fractions were analyzed by (1) reaction with porcine hyperimmune sera raised against the J strain of *M. hyopneumoniae* and (2) staining with amido black. Tryptic fragments stained with amido black that reacted with the hyperimmune sera were analysed by N-terminal amino acid sequencing.

Example B.3 – Cloning of the gene encoding P216

To clone the genes encoding immunoreactive proteins, degenerate oligonucleotide probes were designed from the N-terminal peptide sequences determined above and used to probe *Eco*RI-digested chromosomal DNA by Southern analysis (Southern (1975) *J. Mol. Biol.* 98:503-517). *Eco*RI digested chromosomal DNA from the Beaufort strain was

separated on a 1% agarose column prepared in 491 Prep Cell according to the BioRad Technical Note #2203. Samples from every fifth fraction were blotted to a nylon membrane and probed with degenerate oligonucleotide probes derived from the N-terminal sequences of tryptic fragments. DNA fragments from reactive fractions were incubated with the Klenow fragment and *Pfu* DNA polymerase to generate blunt ends. DNA fragments were ligated into pCR Script™ and transformed into XL10–Gold as outlined in the manufacturer's instructions (Stratagene).

In this way, N-terminal sequence analysis of an X kDa tryptic peptide fragment recognised by porcine hyperimmune generated the sequence ELEDNTKLIAPNIRQ (SEQ ID NO:34). Based on this amino acid sequence, a degenerate oligonucleotide having the sequence 5'-GAA (T/C)T(T/A) GAA GAT AAT AC(C/A/T) AAA TTA ATT GC(T/A) CCT AAT-3' (SEQ ID NO:35) was made and used as a probe to identify a hybridizing fragment of 4.5 kb. The clone containing this 4.5 kilobase fragment was designated p216.

Example B.4 – DNA Sequence Analysis

For sequence analysis, purified plasmid DNA (Qiagen) or PCR product purified from agarose using the BRESA-CLEAN™ kit (Bresatec, Adelaide, Australia) was used. Oligonucleotide primers were obtained commercially (Sigma), and the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems) was used for sequencing reactions. Results were analysed with an Applied Biosystems Model 377 automated sequencer.

Sequence analysis of the cloned fragment in p216 from the Beaufort strain revealed a large ORF that did not significantly match sequences deposited in GenBank. The fragment was the carboxy terminus of a larger ORF as the fragment had a stop codon but no ATG start codon. Additional upstream sequence was obtained by inverse PCR, and the final N-terminal sequence was obtained by PCR using primers designed from strain 232 genomic sequences. The complete ORF (C28-mph545; see, Figure 7) was 5,637 base pairs in length and encoded a protein of 216 kDa designated P216 (C28-MPH545; see, Figure 8). The ORF contained 17 TGA codons, 12 of which appeared in the carboxy terminal 85 kDa.

Blastp analysis of the complete gene sequence revealed near identity with the partial gene sequence YX2 (GenBank Accession No. AF279292) from *M. hyopneumoniae* strain 232 and limited sequence homology with the P97 cilium adhesin (GenBank Accession No. U50901) with 21% identities, 38% positives and 19% gaps (Expect = 4e-18). Comparisons of the nucleotide and derived protein sequences with the database were performed using the package from the University of Wisconsin Genetics Group (GCG) Version 7, accessed via the Australian National Genomic Information Service (ANGIS, University of Sydney) and MacVector (Scientific Imaging Systems, Eastman Kodak Co., New Haven, Conn.).

DNA sequence encoding the P216 homologue from the 232 strain of *M. hyopneumoniae* was obtained as part of a genome-sequencing project. Southern blotting analysis using an oligonucleotide probe from the carboxy terminus showed that the *M. hyopneumoniae* genome contained a single copy of the gene encoding the 216-kDa protein. Blastn analysis with p216 and the *M. hyopneumoniae* genome database also identified a single copy. The protein has 1,879 amino acids, a pI of 8.51, and is highly hydrophilic. A protein motif search using the algorithm Prosite on the ISREC Profilescan server (www.isrec.isb-sib.ch/software/PFSCAN_form.html) identified a bipartite nuclear binding domain (BNBD) between amino acids 1012-1029.

The nucleotide sequence of the *M. hyopneumoniae* p216 gene from strain 232 and the J strain are shown in Figures 7 and 19, respectively.

Example B.5 – Generation of antisera against *M. hyopneumoniae* strain 232

Preparation of porcine hyperimmune serum against *M. hyopneumoniae* is as described in Scarman et al. (1997) *Microbiology* 143:663-673. In brief, *M. hyopneumoniae*-free swines were challenged with a preparation of *M. hyopneumoniae* strain 232 emulsified in Freund's complete adjuvant, and these swines were subjected to a second exposure one month later with the same preparation in Freund's incomplete adjuvant. Serum responses were monitored until an anti-*M. hyopneumoniae* response was confirmed by an enzyme-linked immunosorbent assay (ELISA).

Example B.6 – Generation of P216 polyclonal antisera

To generate monospecific polyclonal antisera to P216, the DNA sequence encoding P216 from strain 232 was examined for the presence of TGA codons, since TGA codons encode tryptophans in *Mycoplasmas*. A region containing no TGA codons and encoding a 30 kDa protein (amino acids 1043-1226) was identified. PCR primers were designed to amplify and clone this region into pCR Script™ forming plasmid p216.1. The cloned fragment was then directionally cloned into pQE9 (Qiagen) by ligation of *Bam*HI- and *Hin*dIII-digested p216.1 DNA to form p216.2. The ligation mixture was transformed into *Escherichia coli* M15[pREP4] according to the manufacturer's instructions (Qiagen). Colony hybridization using the DIG system (Roche) was used to identify transformants containing the proper fragment.

Cultures of the transformants containing p216.2 were grown in LB medium (Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) containing ampicillin (100 μg/mL) and kanamycin (25 μg/mL) at 37°C with shaking. For expression from p216.2, cultures were treated with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) after reaching an OD_{600} of 0.6. After induction for 4 hours, the cells were harvested by centrifugation at 4,000 x*g* for 20 minutes. Purification of the recombinant His-tagged protein was achieved using Ni-NTA resin under denaturing conditions as outlined in the manufacturer's instructions (Qiagen).

Purified recombinant protein was dialysed against PBS containing 5% glycerol and concentrated using polyvinyl-pyrrolidone (Sigma). Approximately 5 mg of purified protein in a volume of 250 μL were emulsified with an equal volume of Freund's incomplete adjuvant (Sigma). The preparation was given subcutaneously to rabbits at two sites and a booster immunization, similarly prepared, was given three weeks later. Serum response against the immunizing antigen was confirmed by immunoblot analysis.

Similarly, rabbit antisera directed against the N-terminal sequence of P216 were generated by immunization with the peptide DFLTNNGRTVLE (SEQ ID NO:36) (amino acids 94-105 of P216) conjugated to keyhole limpet hemocyanin. Rabbit immunizations were performed as described in (Scarman et al. (1997) *Microbiology* 143:663-673).

Example B.7 – Electrophoretic and immunoblot analyses

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis were performed as described by Laemmli (1970) *Nature* 227:680-685 and Towbin et al. (1979) *Proc. Natl. Acad. Sci. USA*, 76:4350-4354, respectively. Analytical electrophoretic gels containing *M. hyopneumoniae* strain 232 proteins were stained with silver (Rabilloud et al. (1992) *Electrophoresis* 13:264-266). Preparative gels were stained with colloidal Coomassie Brilliant Blue G-250 (0.1% Coomassie Brilliant Blue G-250 w/v, 17% w/v ammonium sulfate, 34% methanol v/v, 3% v/v ortho-phosphoric acid). Gels were destained in 1% v/v acetic acid for 1 hour.

Immunoblot analysis was used to determine if P216 is recognised by antibodies elicited during natural infection using swine field sera shown to contain antibodies against *M. hyopneumoniae* (Djordjevic *et al.* (1994) *Vet. Microbiol.* 39:261-273). The 30 kDa recombinant protein representing amino acids 1043-1226 of P216 was used as antigen in these experiments. Other immunoblot analyses included one- and two-dimensional blots of *M. hyopneumoniae* whole cells using swine convalescent sera pools (2D blots) and individual swine sera (1D blots). Swine hyperimmune sera were also used to screen for immunoreactive proteins in one- and two-dimensional immunoblot analyses. Rabbit antisera generated against the 30 kDa recombinant protein and the peptide DFLTNNGRTVLE (SEQ ID NO:36) specific for P130 were used to investigate processing of P216 in one-dimensional immunoblotting experiments as well.

Example B.8 – Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was carried out essentially as described by Guerreiro et al. ((1997) *Mol Plant Microbe Interact* 10:506-516). First dimension immobilized pH gradient (IPG) strips (180 mm, linear and non-linear pH 3-10 and linear pH 4-7; Pharmacia-Biotechnology, Uppsala, Sweden) were prepared for focusing by submersion in rehydration buffer (8 M urea, 0.5% w/v CHAPS, 0.2% w/v DTT, 0.52% w/v Bio-Lyte and a trace of bromophenol) overnight. *M. hyopneumoniae* 232 whole cell proteins (100 μg for analytical gels, 0.5-1.0 mg for preparative gels and immunoblots) were diluted with sample buffer (8 M urea, 4% w/v CHAPS, 1% w/v DTT, 0.8% w/v Bio-Lyte 3-10, 35 mM Tris, and 0.02% w/v bromophenol blue) to a volume of 50 to 100

μl for application to the anodic end of each IPG strip. Isoelectric focusing was run with the Immobiline DryStrip kit in a Multiphor II electrophoresis unit (Pharmacia-Biotechnology) for 200 kVh at 20°C. IEF strips were subsequently prepared for second dimension SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by equilibration in Tris-HCl (0.5 M, pH 6.8) containing 6 M urea, 30% w/v glycerol, 2% w/v sodium dodecyl sulfate (SDS), 2% w/v DTT, and 0.02% bromophenol blue. Equilibrated strips were placed onto Pharmacia ExcelGel gels (T = 12 to 14% acrylamide) for molecular mass separation of *M. hyopneumoniae* proteins on a Multiphor II unit. Electrophoretic conditions consisted of 200 Volts for 1.5 hour followed by 4 hours at 600 Volts. Gels were maintained at 5°C throughout.

Example B.9 – Peptide mass fingerprinting-mass spectrometry

Proteins spots were manually excised and placed in a 96-well microtiter plate. Conditions used for trypsin digestion and for the generation of peptide mass fingerprints are described in Nouwens et al. (2000) *Electrophoresis* 21:3797-3809. A purification step was performed on the tryptic peptides for proteins with poor peptide mass fingerprints as described in Gobom et al. (1999) *J. Mass Spectrom.* 34:105-116. Protein identifications were assigned by comparing the peak lists generated from peptide mass fingerprinting data to a database containing theoretical tryptic digests of *M. hyopneumoniae* strain 232. The Protein-Lynx package (Micromass, Manchester, UK) was used to search databases.

Example B.10 – Image processing

Gels and immunoblots were digitized at 600 dpi with a UMAX PS-2400X lamp scanner using Photoshop 3.0 (Adobe, Mountain View, CA). Spot detection and gel-to-gel protein spot matching were performed with MELANIE II software (BioRad, Hercules, CA) run under OpenWindows 3.0. Apparent molecular masses were determined by co-electrophoresis with protein standards (Pharmacia-Biotechnology).

Example B.11 – Results of two-dimensional electrophoresis and peptide mass fingerprinting analysis

Analyses of two-dimensional electropherograms identified two clusters of spots that tracked along the pI gradient in an unusual fashion. Peptide mass fingerprinting analysis of spots within each of the clusters showed that the spots had identical mass fingerprints and were thus derived from the same molecule. Cluster 1 with an approximate mass of 130 kDa was mapped to the N-terminal region of P216 from the genome sequence of *M. hyopneumoniae* strain 232. Cluster 2 of approximately 85 kDa mapped to the carboxy terminus of the same ORF. The proteins were designated P130 and P85, respectively. The pI of cluster 1 ranged from 9.5 to 8.0, while the pI of cluster 2 ranged from 9.0 to 6.5. Mass spectrometric analysis indicated that P216 was cleaved between amino acids 1004 and 1090 generating the two fragments of 130 and 85 kDa.

Example B.12 – Results of immunoblot analysis

Two-dimensional immunoblots reacted with porcine hyperimmune sera revealed a complex pattern of spots two of which corresponded to P130 and P85. P85 was also strongly recognized by a pool of convalescent sera showing that it was an important antigen during disease. To investigate this further, a 30-kDa region spanning amino acids 1042-1226 in P85 was expressed, purified by nickel-affinity chromatography, and blotted onto PVDF membrane. Individual convalescent sera from swines known to be positive in a *M. hyopneumoniae*-specific ELISA reacted with the 30-kDa protein confirming that P216 is an important molecule recognized by the host immune response during the normal course of infection. Antibodies raised to a 30-kDa peptide spanning amino acids 1042-1226 reacted solely with the 85 kDa cleavage product suggesting that cleavage occurred between amino acids 1004 and 1042. Sera raised to the N-terminal peptide of P216 recognized only P130

Example B.13 – Posttranslational processing of P216 among different strains of *M. hyopneumoniae.*

To investigate fragment patterns of P216 in different *M. hyopneumoniae* strains, immunoblot analysis was performed with the anti-P130 N-terminal peptide and anti-P30 antisera. Antibodies raised against the N-terminal peptide recognized P130 and several lower molecular mass peptides in one-dimensional immunoblots of whole cell lysates of J

and 232 strains. The pattern of proteins recognised by this antisera was different between the two strains. Antisera raised against the 30-kDa peptide strongly recognised an 85-kDa antigen in both J and 232 strains, but also reacted with a number of weakly reactive proteins. Similarly, the pattern recognised with the anti-30-kDa sera was different between J and 232.

To determine if different post-translational cleavage events were occurring among other strains of *M. hyopneumoniae,* a collection of strains from different geographic origins were examined by immunoblot. Anti-30 kDa sera reacted strongly to an 85-kDa antigen and other proteins of lower molecular mass in immunoblots of whole cell lysates from different strains of *M. hyopneumoniae.* These strains represented isolates recovered from different geographic locations within Australia and from different countries including the USA, Great Britain and France. The anti-P30 sera, however, did not react against antigens in immunoblots of whole cell lysates of related porcine *Mycoplasmas*, e.g. *Mycoplasma hyorhinis* and *Mycoplasma flocculare,* suggesting that P216 is a *M. hyopneumoniae*-specific antigen. Convalescent sera from different swines also recognized purified recombinant P30 indicating that P216 is expressed *in vivo.*

Example B.14 – Surface localization studies

Several approaches were taken to determine if P216 and its cleavage products were associated with the outer membrane surface. These included trypsin digestion and cell surface biotinylation.

For trypsin digestion studies, all solutions and *M. hyopneumoniae* cell stocks were pre-equilibrated at 37°C. *M. hyopneumoniae* cells (200 mg/mL in PBS) were aliquoted (300 μL) into sterile eppendorf tubes at 37°C and trypsin was added to a final concentration ranging from 0.1 - 1000 μg/mL. The suspensions were inverted gently and incubated at 37°C for 20 minutes. Immediately after incubation, the cells were lysed in Laemmli buffer, heated at 95°C for 10 minutes and analysed by SDS PAGE and immunoblotting. Trypsin digested both P85 and P130 in a concentration dependent manner, but did not digest the intracellular enzyme lactate dehydrogenase, a control for spontaneous lysis of cells (Strasser et al. (1991) *Infect. Immun.* 59:1217-22). This

suggests that both portions of P216 are surface accessible and sensitive to trypsin digestion.

To further clarify this, surface biotinylation of *M. hyopneumoniae* was performed. The method described by Meier et al. ((1992) *Anal. Biochem.* 204:220-226) was used with the following modifications. All solutions were pre-chilled at 4°C and all manipulations were performed on ice. *M. hyopneumoniae* pellets (200 mg wet weight) were resuspended in 4 mL of BOS buffer (10 mM sodium tetraborate in 0.15 M NaCl, pH 8.8). Immediately after the addition of 5 µL of NHS-biotin (10 mg/mL in dimethylsulfoxide), the reaction was allowed to proceed for 1 to 8 minutes with swirling. To determine the most suitable reaction time, aliquots were removed at 1-minute intervals for 15 minutes. A reaction time of 5 minute was chosen for all subsequent studies except where noted. Biotinylation was stopped with the addition of 2 mL of 0.1 M NH_4Cl that served to saturate unbound NHS-biotin. Cells were harvested by centrifugation (8,500 xg, 10 minutes) and washed twice in TKMS buffer (25 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$ and 0.15 M NaCl in PBS). The products were resolved by two-dimensional electrophoresis.

Both P130 and P85 were readily biotinylated, confirming that all parts of P216 were surface accessible.

Example B.15 – Triton X-100 and X-114 extractions

Integral membrane proteins from 200 mg wet weight of whole cells were extracted with TX-114 essentially as described by Bordier ((1981) *J. Biol. Chem.* 182:1356-1363). The resultant aqueous and detergent phases were collected and analysed by SDS-PAGE and immunoblotting. The phase partitioning activity of Triton X-114 causes separation of hydrophobic molecules into the detergent phase. When treated with Triton X-114, P85 remained in the insoluble pellet consisting of complex high molecular weight structures that (1) were membrane associated and (2) lacked the solubility of normal cytosolic proteins.

For Triton X-100 extraction, pelleted *M. hyopneumoniae* (strains J and Beaufort) cells (200 mg wet weight) were resuspended in 10 mL of TS buffer containing 1 mM phenylmethylsulfonyl fluoride. Proteins were extracted by the addition of 2% Triton X-

100 (Amersham Pharmacia Biotechnology) and incubated at 37°C for 30 minutes as described in Stevens and Krause ((1991) *J. Bacteriol.* 173:1041-1050). Briefly, *M. hyopneumoniae* cell suspensions were centrifuged (14,000 xg, 30 min) at 4°C. The aqueous phase was removed and the pellet was re-extracted as described above. The insoluble pellet and both aqueous phases were analysed by SDS-PAGE and immunoblotting using anti-30 kDa and sera raised against the peptide DFLTNNGRTVLE (SEQ ID NO:36).

With Triton X-100 fractionation, high molecular weight cytoskeletal-like proteins remain insoluble, but phase partitioning does not occur. When treated with Triton X-100, P85 partitioned primarily to the aqueous detergent-containing phase, but about 30% remained in the pellet. These data indicate that P216 may form extracellular oligomeric structures. The presence of coiled coil domains in both fragments of P216 also supports this hypothesis.

C. P97 STUDIES

Example C.1 - Bacterial strains and plasmids

M. hyopneumoniae strains 232 (virulent parental strain), 232_91.3 (high adherent clone), 232_60.3 (low adherent clone), and J type strain (NCTC 10110) were grown in modified Friis broth and harvested as described by Zhang et al. ((1995) *Infect Immun* 63:1013-1019) and Djordjevic et al. ((1994) *Vet Microbiol* 39:261-273), respectively. All broth media were filter sterilized through 0.22 μm filters, which removed the majority of particulate matter. Mycoplasmas were harvested by centrifugation and extensively washed to remove remaining medium contaminants. *Escherichia coli* TOP10 containing pISM405 was grown on Luria Bertani (LB) agar or in LB broth (Sambrook et al., 1989) containing 100 μg ml^{-1} ampicillin. Isopropyl-β-D-thiogalactopyranoside (IPTG) induction was carried out by the addition of IPTG to a final concentration of 1 mM. Bacterial cultures were routinely grown at 37°C and liquid cultures were aerated by shaking at 200 rpm.

Example C.2 - Construction and expression of adhesin fusion protein

Hexa-histidyl P97 fusion proteins were constructed using the pTrcHis (Invitrogen, Carlsbad, CA) cloning vector. Primers FMhp3 (5'-GAA CAA TTT GAT CAC AAG ATC CTG AAT ATA CC-3' (SEQ ID NO:37)) and RMhp4 (5'-AAT TCC TCT GAT CAT TAT TTA GAT TTT AAT TCC TG-3' (SEQ ID NO:38)) were used to amplify a 3013 bp fragment representing base pairs 315-3321 of the gene sequence containing amino acids 105-1107. The fragment was digested with *BclI* (underlined sequence) and inserted into the *Bam*HI site of vector pTrcHisA. A construct with the proper fragment orientation was identified by restriction digests. The resulting 116-kDa recombinant P97-polyhistidine fusion protein contained the R1 and R2 repeat regions as well as the major cleavage site at amino acid 195 in the P97 sequence.

Example C.3 – Antisera

The Mab F1B6 has been described (Zhang et al. (1995) *Infect. Immun.* 63:1013-1019). Mab F1B6 binds to the R1 region of the cilium adhesin that has at least 3 repeat sequences (Minion et al. (2000) *Infect. Immun.* 68:3056-3060). Peptides with sequences TSSQKDPST (ΔNP97) (SEQ ID NO:39) and VNQNFKVKFQAL (NP97) (SEQ ID NO:40) were used to raise antibodies against P97/P66 and P22, respectively. The peptides were bound to keyhole limpet hemocyanin with the Pierce Imjet Maleimide Activated Immunogen Conjugation Kit (Pierce Chemical Co., Rockford, IL). These conjugates were then used to generate mouse hyperimmune antisera by the method of Luo and Lin ((1997) *BioTechniques* 23:630-632). The resulting antisera were tested by enzyme linked immunosorbent assay (ELISA) using ovalbumin-peptide conjugate and purified recombinant P97 antigens, and by immunoblot with the recombinant P97 antigen. Antiserum raised against the C-terminal 28 kDa (R2 serum) of the cilium adhesin of strain J has been described (Wilton et al. (1998) *Microbiology* 144:1931-1943). Mouse Mab 2B6-D4 raised against human fibronectin was purchased commercially (BD Biosciences, Pharmingen) as was alkaline phosphatase conjugated goat anti-mouse Ig(H+L) antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL). Goat anti-mouse IgG + IgM labeled with 10 nm colloidal gold particles (EY Laboratories, Inc., San Mateo, CA) was used in immunogold electron microscopy studies.

Example C.4 – Immunoblot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis was performed as described by Laemmli ((1970) *Nature* 227:680-685) and Towbin et al. ((1979) *Proc. Natl. Acad. Sci. USA.* 76:4350-4354), respectively. Proteins were transferred to PVDF membranes (Micron Separations, Inc.). For the media control experiments, purified recombinant P97 was incubated with fresh and spent Friis media. Spent media was prepared from an early log phase culture that had been centrifuged and filtered through a 0.1 μm filter. Purified recombinant P97 (2.5 μg) in 20 μl phosphate buffered saline was diluted 1:1 in fresh or spent media and incubated overnight at 37°C. Ten μl of the mixture were the loaded onto SDS-PAGE gels, blotted to nitrocellulose and developed with F1B6 Mab. For ligand blotting, PVDF blots were transferred, blocked and washed as described previously (Wilton et al. (1998) *Microbiology* 144:1931-1943). Blots were exposed to human fibronectin (5 μg ml^{-1}) dissolved in TS buffer (TS buffer: 10 mM Tris-HCl, pH 7.4; 150 mM NaCl) for 1.5 h, washed, and exposed to 0.4 μg ml^{-1} anti-human fibronectin Mabs for 1 h at room temperature. Blots were washed and developed as described above.

Example C.5 – Trypsin treatment of *M. hyopneumoniae*

M. hyopneumoniae cells (0.5 g) were treated with trypsin essentially as described previously (Wilton et al. (1998) *Microbiology* 144:1931-1943). Briefly, trypsin was added to cell suspensions of *M. hyopneumoniae* at 0, 0.3, 0.5, 1.0, 3.0, 10, 50, 300, and 500 μg ml^{-1} at 37°C for 15 min. Immediately after incubation, cell suspensions were lysed in Laemmli buffer and heated to 95°C for 10 min. Lysates were analysed by SDS-PAGE and immunoblotting using F1B6 Mab.

Example C.6 – Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2-DGE) was carried out essentially as described by Cordwell et al. ((1997) *Electrophoresis* 18:1393-1398). First dimension immobilized pH gradient (IPG) strips (180 mm, linear pH6-11; Amersham Phamracia Biotech, Uppsala, Sweden) were prepared for focusing by submersion in 2-DGE

compatible sample buffer (5 M urea, 2 M thiourea, 0.1% carrier ampholytes 3-10, 2% w/v CHAPS, 2% w/v sulfobetaine 3-10, 2mM tributyl phosphine (TBP; Bio-Rad, Hercules USA)) overnight. *M. hyopneumoniae* whole cell protein (250 μg)) was diluted with sample buffer to a volume of 100 μl for application to the anodic end of each IPG strip via an applicator cup. Isoelectric focusing was performed with a Multiphor II electrophoresis unit (Amersham Pharmacia Biotech) for 85 kVh at 20°C. IPG strips were detergent exchanged, reduced and alkylated in buffer containing 6 M urea, 2% SDS, 20% glycerol, 5 mM TBP, 2.5% v/v acrylamide monomer, trace amount of bromophenol blue dye and 375 mM Tris-HCl (pH 8.8) for 20 minutes prior to loading the IPG strip onto the top of an 8-18% T, 2.5% C (piperazine diacrylamide) 20 cm x 20 cm polyacrylamide gel. Second-dimension electrophoresis was carried out at 4°C using 3mA/gel for 2 hours, followed by 20mA/gel until the bromophenol blue dye had run off the end of the gel. Gels were fixed in 40% methanol, 10% acetic acid for 1 hour and then stained overnight in Sypro Ruby (Molecular Probes, Eugene, OR). Images were acquired using a Molecular Imager Fx (Bio-Rad). Gels were then double-stained in Coomassie Blue G-250.

Example C.7 – Post-separation analyses

Protein spots were excised from gels using a sterile scalpel and placed in a 96 well tray (Gobom et al. (1999) *J. Mass. Spectrom.* 34:105-116). Gel pieces were washed with 50 mM ammonium bicarbonate/100% acetonitrile (60:40 v/v) and then dried in a Speed Vac (Savant Instruments, Holbrook, NY) for 25 min. Gel pieces were then hydrated in 12 μl of 12 ng μl^{-1} sequencing grade modified trypsin (Promega, Madison, WI) for 1 h at 4°C. Excess trypsin solution was removed and the gel pieces immersed in 50 mM ammonium bicarbonate and incubated overnight at 37°C. Eluted peptides were concentrated and desalted using C_{18} Zip-Tips™ (Millipore Corp., Bedford, MA). The peptides were washed on a column with 10 μl 5% formic acid. The bound peptides were eluted from the Zip-Tip™ in matrix solution (10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid [Sigma] in 70% acetonitrile) directly onto the target plate. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) mass spectra were acquired using either a PerSeptive Biosytems Voyager DE-STR (Framingham, MA)

or a Micromass TofSpec2E (Micromass, Manchester UK). Both instruments were equipped with 337 nm nitrogen lasers. All spectra were obtained in reflectron/delayed extraction mode, averaging 256 laser shots per sample. Two-point internal calibration of spectra was performed based upon internal porcine trypsin autolysis peptides (842.5 and 2211.10 $[M+H]^+$ ions). A list of monoisotopic peaks corresponding to the mass of generated tryptic peptides was used to search a modified translated version of the *M. hyopneumoniae* genome. Successful identifications were based on the number of matching peptide masses and the percentage sequence coverage afforded by those matches. N-terminal Edman sequencing was performed as previously described (Nouwens et al. (2000) *Electrophoresis* 21:3797-3809).

Example C.8 – Immunoelectron microscopy

M. hyopneumoniae strain 232 cells were grown to mid log phase, pelleted by centrifugation and washed with phosphate buffered saline (PBS). The final cell pellets were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C overnight. The pellets were washed three times with 0.1 M sodium cacodylate buffer, 15 min between changes and post fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h at room temperature. The pellets were then washed with distilled water, passed through an acetone series and embedded in Embed 812 and Araldite (Electron Microscopy Sciences, Fort Washington, PA). Thin sections (80-90 nm) were then washed six times with TS buffer, and reacted with F1B6 ascites fluid (diluted 1:50), anti-ΔNP97 ascites fluid (diluted 1:10), anti-NP97 ascites fluid (diluted 1:10), or mouse anti-human fibronectin (diluted 1:25) overnight at 4°C. The grids were washed five times with TS buffer and then reacted with goat anti-mouse IgG + IgM labeled with 10 nm colloidal gold particles (EY Laboratories, Inc.) diluted 1:25 for 30 min at room temperature. The cells were then washed 5 times with TS buffer, dried, contrasted with osmium vapors for 2 min, and stained with uranyl acetate-lead citrate. The sections were examined on a Hitachi 500 at 75 kV.

For tracheal sections, mycoplasma-free pigs were inoculated intratracheally with *M. hyopneumoniae* strain 232. At 10 and 21 days, pigs were sacrificed, tracheas were removed and 1 cm blocks of tissue fixed with 1% glutaraldehyde overnight, dehydrated

in an acetone series, and embedded as above. Thick (1-2 μm) sections were stained with methylene blue polychrome and examined by microscopy for regions containing ciliated epithelium. Thin sections (80-90 nm) were then prepared for labeling. The sections were pretreated with ammonium chloride (1%) for 1 h, 0.05 M glycine in PBS for 15 min, blocked for 30 min in 2% fish gelatin + 2% bovine serum albumin in TS buffer (10 mM Tris, 100 mM NaCl, pH 7.5). Primary antibodies were diluted in TS buffer and reacted with sections for 30 min at room temperature. The sections were washed six times with TS buffer, and then incubated with goat anti-mouse IgG + IgM labeled with 10 nm gold particles (diluted 1:2) for 15 min at room temperature. Both primary antibodies and the conjugate were diluted and centrifuged briefly (12,000 xg for 5 min) prior to use. The sections were then washed six times with TS buffer, dried, contrasted with osmium vapors for 2 min, and stained with uranyl acetate-lead citrate. The sections were examined on a Hitachi 500 at 75 kV.

Example C.9 – Fibronectin binding assay

Immunlon 2 (Dynatech Laboratories, Inc.) 96 well plates were coated with 100 μl of human fibronectin (Sigma, F 0895) at a concentration of 5 μg ml^{-1} in 0.1 M sodium carbonate. Plates were incubated at 4°C overnight, washed three times with PBS, and blocked with 1% bovine serum albumin in PBS for 2 hr. The plates were then incubated with purified recombinant P97 with or without inhibitor at a concentration of 10 μg ml^{-1}. Inhibitors tested were intact human fibronectin, 45-kDa proteolytic fragment of fibronectin (Sigma, F 0162), 30-kDa proteolytic fragment of fibronectin (Sigma, F 9911) and engineered RGD polymer (Sigma, 5022). They were added to Eppendorf tubes with purified recombinant P97 (10 μg ml^{-1}) at concentrations of 37.5 μg ml^{-1}, 7.5 μg ml^{-1}, and 1.5 μg ml^{-1} and incubated at 37°C for 1 hr. The recombinant P97 plus inhibitor was then transferred to a fibronectin coated plate, which was then incubated at 37°C for 2 hr. Binding of P97 to fibronectin was assessed by ELISA with Mab F1B6. Optical density at 405 nm was indicative of P97 binding to fibronectin-coated wells. Three replicates per treatment were assayed from three different experiments. Statistical differences were determined by the General Linear Model with a linear contrast based on pooled variances.

Example C.10 – Results of two-dimensional gel electrophoresis and mass spectrometry

Previous studies have demonstrated that the gene product for the cilium adhesin of strain 232 (126-kDa preprotein, 1036 amino acids) undergoes a cleavage event at amino acid 195 to yield what was once thought to be the "mature" molecule (Hsu et al. (1997) *J. Bacteriol.* 179:1317-1323). During peptide mass mapping studies of J strain proteins, four spots of 22, 28, 66 and 94 kDa (subsequently referred to as P22, P28, P66 and P94, respectively) were identified that represented different fragments of the adhesin. The N-terminal sequences for these proteins allowed unequivocal alignment with the cilium adhesin preprotein. P94 of strain J, the homologue of P97 in strain 232, mapped to a region that begins immediately downstream of amino acid 195 until the end of the ORF. Two closely spaced proteins at 66 kDa had identical mass maps and corresponded to a region beginning immediately downstream of amino acid 195 of the adhesin and ending near the R1 repeat. N-terminal sequence analysis of P66 showed a sequence of ADEKTSS (SEQ ID NO:41) that is identical to that of P94. Immunoblotting results using Mab F1B6 confirmed that P66 contains R1. Thus, the cleavage event must occur immediately downstream of the R1 repeat region. These data suggest that a fragment approximately 28 kDa in size had been removed from the C-terminus in some, but not all of the P94 molecules. This observation was confirmed when a 28-kDa fragment was identified that mapped to the C-terminus of P94. Also, one and two-dimensional immunoblots of J strain proteins probed with antisera raised against a recombinant 28-kDa protein containing R2 but not R1 (Wilton et al. (1998) *Microbiology* 144:1931-1943) recognised both P28 and P94 proteins. Previously, it was shown that antisera raised against a 28-kDa C-terminal recombinant peptide of the adhesin recognised the mature form of this antigen (93-97 kDa) in different strains of *M. hyopneumoniae* and a 28-kDa fragment only in strain J (Wilton et al. (1998) *Microbiology* 144:1931-1943). Tryptic peptide mass mapping showed that peptides from P22 mapped to the first 190 amino acids of the 123-kDa adhesin preprotein. The N-terminal sequence of P22 (SKKSKTF (SEQ ID NO:42)) aligned to amino acids 2-8 in the N-terminus of the 123 kDa pre-

protein suggesting that cleavage of the hydrophobic leader peptide (amino acids 8-22) is not necessary for translocation of the cilium adhesin across the membrane.

Comparative peptide mass mapping studies of strain 232 identified two spots of 70 and 97 kDa, subsequently identified as P70 and P97, respectively. Mass maps representative of P97 corresponded to a region beginning immediately downstream of amino acid 195 until the end of the ORF and corresponded to the most abundant product of the 232 strain adhesin gene (Zhang et al. (1995) *Infect. Immun.* 63:1013-1019). Interestingly, mass maps representative of P70 corresponded to a region beginning immediately downstream of amino acid 195 and ending near the R1 repeat, a map that was virtually identical to P66 in strain J. The presence of six extra copies of the R1 repeat is the most likely explanation for the difference in masses between P66 and P70 in strains J and 232, respectively. Consistent with these data, immunoblots probed with antisera raised against a recombinant 28-kDa protein containing R2 but not R1 (Wilton et al. (1998) *Microbiology* 144:1931-1943) recognized P97 but not P70 or P28. Furthermore, P28 or P22 could not be identified on 2D gels of 232 proteins resolved by 2D gel electrophoresis in regions where they were identified in strain J. This variation was not due to differences in sequence since P22 sequences were identical in the two strains. This was not true for the P28 sequences, however. The predicted mass and pI for P28 from strain 232 was 24.6 kDa and 5.88, respectively, and for P28 from strain J, it was 26.0 kDa and 8.39. It was possible that P28 was not found in strain 232 because of the change in pI causing a shift in the gel location of the protein. It was also possible that additional cleavage of P22 occurred in strain 232 that did not in strain J.

To rule out the possibility that cleavage resulted from a proteolytic activity in the media used for culturing *M. hyopneumoniae*, purified recombinant P97 was incubated with fresh and spent medium and then examined for proteolytic cleavage by immunoblot. Because the medium contained 20% swine serum, large quantities of swine immunoglobulins were present in the protein samples causing some background staining with the anti-mouse conjugate. It was still clear, however, that neither fresh nor spent medium contained proteolytic activity capable of cleaving recombinant P97 after 12 hours of incubation at 37°C. Thus, cleavage of the cilium adhesin was mediated by

mycoplasma-encoded activities and was not due to porcine serum or other medium components.

Example C.11 – Trypsin sensitivity of R1-containing cleavage products

Immunoblot analyses of strain J and 232 cells digested with different concentrations of trypsin was used to investigate the cellular location of R1-containing cleavage fragments. The F1B6 Mab typically recognised proteins with masses of 35, 66, 88, 94, and 123 kDa in strain J and a similar pattern was observed for strain 232. Exposure of intact *M. hyopneumoniae* to concentrations of trypsin ranging from 0.1-10 $\mu g\ ml^{-1}$ showed a gradual loss of the higher mass proteins. Concentrations between 10 and 50 $\mu g\ ml^{-1}$ resulted in the loss of all the immunoreactive proteins (except one of 35 kDa) indicating that R1-containing adhesin fragments are surface accessible. The pattern of digestion of R1-containing adhesin fragments was consistent in repeat experiments except that the 35 kDa fragment was not reliably resistant to trypsin at concentrations above 10 $\mu g\ ml^{-1}$. Identical blots reacted with antisera raised to recombinant *M. hyopneumoniae* lactate dehydrogenase (previously shown to reside cytosolically) (Strasser et al. (1991) *Infect. Immun.* 59:1217-1222) and to antisera raised to recombinant fragments of pyruvate dehydrogenase subunits A and D showed that these proteins remained detectable with trypsin concentrations up to 500 $\mu g\ ml^{-1}$. In control experiments where lysed cells were exposed to trypsin, lactate dehydrogenase and pyruvate dehydrogenase subunit D were rapidly degraded.

Example C.12 – Results of immunogold electron microscopy

Transmission electron microscopy studies have shown that high and low adherent strains of *M. hyopneumoniae* differ in their outer membrane structure. High adherent clones possessed fibrils on the outer surface that appeared to interconnect to adjacent cells; these fibrils were rarely observed in low adherence clones (Young et al. (1994) Isolation and characterization of high and low adherent clones of *Mycoplasma hyopneumoniae*. In *IOM Letters. 10th International Congress of the International Organization for Mycoplasmology*. Vol. 3 Bordeaux, France, pp. 684-685). Antisera generated against specific regions of the adhesin enabled analysis of cleavage *in vivo*

using immunogold electron microscopy. Virulent strain 232 was used in these studies because these results would have the most impact on understanding pathogenic mechanisms. R1-specific Mab F1B6 and antisera raised to peptides TSSQKDPST (ΔNP97 antiserum) (SEQ ID NO:39) and VNQNFKVKFQAL (NP97 antiserum) (SEQ ID NO:40) were used in these studies. The Mab F1B6 remained associated with the mycoplasma membrane, but not intimately associated with the cell confirming a previous report (Zhang et al. (1995) *Infect. Immun.* 63:1013-1019) and the trypsin studies above. ΔNP97 antiserum showed that this portion of the molecule is located distal to the membrane in association with extracellular material of unknown composition. In some instances, the antibodies seemed to define fibrial-like structures still attached to the mycoplasma cell membrane. NP97 antibodies clustered in aggregates to cytosolic locations, intimately to the membrane surface, and were also observed at sites distant from the extracellular surface of the cell membrane.

Example C.13 – Fibronectin binding results

Since cleavage of the cilium adhesin occurs at amino acid position 195 (Hsu et al. (1997) *J. Bacteriol.* 179:1317-1323), it was not readily apparent how the remaining adhesin could remain associated with the cell and direct binding to porcine cilia. Immunogold studies showed that all cilium binding R1 epitopes remained cell associated in the absence of the hydrophobic N-terminus sequence, but apparently are not inserted directly into the membrane. This is not surprising since no other region of the protein has sufficient hydrophobicity to direct membrane insertion (Hsu et al. (1997) *J. Bacteriol.* 179:1317-1323). The possibility that other proteins may play a role in bridging R1-containing protein fragments of the cilium adhesin to the membrane through protein-protein interactions was examined. Analysis of the predicted protein sequence of the 123 kDa adhesin preprotein with the computer program COILS (http://www.ch.embnet.org) revealed that the protein contained three coiled coil domains. One of these resided between amino acids 180-195 in P22 (14-, 21- and 28-amino acid window settings) and two were located in P97 between amino acids 367-387 (window setting 14) and 780-805 (window setting 14 and 21). These domains are known to mediate protein-protein interactions. In addition, it was thought that the R1 and R2 domains might also play a

role in interactions with other proteins. One obvious protein to test was fibronectin, a protein found in abundance throughout the host and shown to participate in other bacterial-host interactions (Probert et al. (2001) *Infect. Immun.*69:4129-4133; Talay et al. (2000) *Cell Microbiol.* 2:521-535; Rocha and Fischetti (1999) *Infect. Immun.* 67:2720-2728; and Schorey et al. (1996) *Mol. Microbiol.* 21:321-329).

Ligand blotting studies confirmed that recombinant P97 bound porcine fibronectin. Other fibronectin binding proteins were also identified in lysates of *M. hyopneumoniae* low (lane 1) and high (lane 2) adherent variants of strain 232 and in strain J (lane 3). The low and high adherent strains of 232 differed by the absence of a fibronectin-binding band at approximately 50 kDa, which was also present in strain J.

Fibronectin binding assays with human fibronectin and purified recombinant cilium adhesin were also performed. Maximum inhibition occurred with the engineered RGD domain at all three concentrations tested ($p < 0.001$). Inhibition also occurred with intact fibronectin ($p < 0.001$) as expected. Interestingly, the 45-kDa purified fragment of fibronectin enhanced binding at the highest concentration tested.

To investigate the role(s) fibronectin might play in the binding of *M. hyopneumoniae* to porcine respiratory epithelial cells, anti-fibronectin antibodies were applied to lung sections showing *M. hyopneumoniae* strain 232 in close association with respiratory epithelial cilia. Gold particles were localised in regions where *M. hyopneumoniae* cells were intimately associated with cilia, on the surface of cilia and on the surface of *M. hyopneumoniae* cells.

D. DETECTION OF INFECTION AND IMMUNOGENIC COMPOSITIONS

Example D.1 – Detection of *M. hyopneumoniae* infection in swine

The polypeptides displaying *M. hyopneumoniae* antigenicity of this invention may be used in methods and kits designed to detect the presence of *M. hyopneumoniae* infection in swine herds and therefore to recognize swine in a herd which have been infected by this bacteria. For example, the antigens produced by hosts transformed by recombinant nucleic acid molecules of this invention, or antibodies raised against them, can be used in RIA or ELISA for these purposes. In one type of radioimmunoassay, antibody against one or more of the antigens of this invention, raised in a laboratory

animal (e.g., rabbits), is attached to a solid phase, for example, the inside of a test tube. Antigen is then added to the tube to bind with the antibody.

A sample of swine serum, taken from 1 of each 10 to 20 swine per herd, together with a known amount of antigen antibody labeled with a radioactive isotope, such as radioactive iodine, is then added to the tube coated with the antigen-antibody complex. Any antigen (a marker for *M. hyopneumoniae* infection) antibody in the swine serum will compete with the labeled antibody for the free binding sites on antigen-antibody complex. Once the serum has been allowed to interact, the excess liquid is removed, the test tube washed, and the amount of radioactivity measured. A positive result, i.e., that the tested swine's serum contains *M. hyopneumoniae* antibody, is indicated by a low radioactive count.

In one type of ELISA test, a microtiter plate is coated with one or more antigens of this invention and to this is added a sample of swine serum, again, from 1 in every 10 or 20 swine in a herd. After a period of incubation permitting interaction of any antibody present in the serum with the antigen, the plate is washed and a preparation of antigen antibodies, raised in a laboratory animal and linked to an enzyme label, is added, incubated to allow reaction to take place, and the plate is then rewashed. Thereafter, enzyme substrate is added to the microtiter plate and incubated for a period of time to allow the enzyme to work on the substrate, and adsorbance of the final preparation is measured. A large change in adsorbance indicates a positive result, i.e., the tested swine serum had antibodies to *M. hyopneumoniae* and was infected with that bacteria.

Example D.2 – Immunogenic compositions

Standard methods known to those skilled in the art may be used in preparing immunogenic compositions of polypeptides and nucleic acids of the present invention for administration to swine. For example, the polypeptide of choice may be dissolved in sterile saline solution. For long-term storage, the polypeptide may be lyophilized and then reconstituted with sterile saline solution shortly before administration. Prior to lyophilization, preservatives and other standard additives such as those to provide bulk, e.g., glycine or sodium chloride, may be added. A compatible adjuvant may also be administered with the composition.

In addition, compositions can be prepared using antibodies raised against the polypeptides of this invention in laboratory animals, such as rabbits. This "passive" vaccine can then be administered to swine to protect them from *M. hyopneumoniae* infection. Direct incorporation of nucleic acid sequences into host cells may also be used to introduce the sequences into animal cells for expression of antigen *in vivo*.

The above description, drawings and examples are only illustrative of preferred embodiments that achieve the objects, features and advantages of the present invention. It is not intended that the present invention be limited to the illustrated embodiments. Any modification of the present invention that comes within the spirit and scope of the following claims should be considered part of the present invention.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.